Developing An Effective Microbial Formulation For Biodegradation Of Pineapple Waste Via Solid State Fermentation

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Table of Contents

Acknowledgement i

Table of Contents ..... ii-iv

List of Abbreviations v

List of Tables vi

List of Figures ..... vii-viii

Abstract 1

1.0 Introduction 2-3

2.0 Literature Review

2.1 Pineapple Industry and its By-product 4

2.2 *Bacillus licheniformis* 5

2.3 *Bacillus amyloliquefaciens* 5-6

2.4 *Trichoderma harzianum* 6

2.5 *Ananas comosus* 7

2.6 Solid State Fermentation (SSF) 7-8

3.0 Method and Methodology

3.1 Microorganism preparation 9

3.2 Inoculum preparation

3.2.1 Bacterial inoculum preparation 9

3.2.2 Fungus spore preparation 9-10

3.3 Pineapple waste preparation 10

3.4 Solid State Fermentation (SSF) 10

3.5 Analytical method

3.5.1 Biomass Dry Weight 11

3.5.2 Protein concentration 11

3.5.3 Carbon content 11-12

3.5.4 Nitrogen content 12

3.5.5 Digestion and washing step 12

3.5.5.1 Lignin 12-13

3.5.5.2 Reducing sugar and glucose 13

3.5.5.3 Cellulose and hemicellulose calculation 13-14

4.0 Results and discussion

4.1 Preparation of culture

4.1.1 *Trichoderma harzianum* culture 15

4.1.2 *B.amyloliquefaciens* and *B.licheniformis* culture 15-16

4.2 Lignin degradation 16-19

4.3 Cellulose and hemicellulose degradation 19-20

4.3.1 Cellulose degradation 20-25

4.3.2 Hemicellulose degradation 22-25

4.4 Glucose and reducing sugar concentration 25

4.4.1 Glucose concentration 25-29

4.4.2 Reducing sugar concentration 27-29

4.5 Biomass dry weight 29-31

4.6 Protein content 31-34

4.6 Carbon to nitrogen ratio 34-35

5.0 Conclusion 36

6.0 References 37-40

7.0 Appendices

List of Abbreviations

SSF	Solid State Fermentation
CMC	Carbomethylcellulase
PDA	Potato Dextrose Agar
LB	Luria Broth
DNS	Di-nitrosalicyclic
UV	Ultraviolet
$MgSO_4$	Magnesium Sulphate
KH_2PO_4	Monopotassium Phosphate
K_2HPO_4	Dipotassium Phosphate
NH_4NO_3	Ammonium Nitrate
$CaCl_2$	Calcium Chloride
$FeCl_2$	Ferrous Chloride
H_2SO_4	Sulphuric Acid
OD	Optical Density

List of Tables

Table 1	Experimental set with inoculation of different strain and combination of microorganisms	9
Table 2	Biomass dry weight calculation	10
Table 3	Carbon percentage calculation	11
Table 4	Lignin residue percentage calculation	12
Table 5	Carbon to Nitrogen ratio	32
Table 6	Summary of all analysis conducted on the substrate after fermentation with the co-cultivation of *T.harzianum* UMAS SD17, *B.amyloliquefaciens* UMAS 1002 and *B.licheniformis* UMAS P7	35

List of Figures

Figure 1	Lignin degradation over days via fermentation with mono culture cultivation	16
Figure 2	Lignin degradation over days via fermentation with mixed culture cultivation	16
Figure 3	Lignin degradation over days.	17
Figure 4	Cellulose degradation over days via fermentation with mono culture cultivation.	19
Figure 5	Cellulose degradation over days via fermentation with mixed culture cultivation	19
Figure 6	Cellulose degradation over days	20
Figure 7	Hemicellulose degradation over days via fermentation with mono culture cultivation	22
Figure 8	Hemicellulose degradation over days via fermentation with mixed culture cultivation	22
Figure 9	Hemicellulose degradation over days	23
Figure 10	Glucose concentration over days via fermentation with mono culture cultivation	24
Figure 11	Glucose concentration over days via fermentation with mixed culture cultivation	24
Figure 12	Glucose concentration over days	25
Figure 13	Reducing sugar concentration over days via fermentation with mono culture cultivation	26
Figure 14	Reducing sugar concentration over days via fermentation	27

	with mixed culture cultivation	
Figure 15	Reducing sugar concentration over days	27
Figure 16	Biomass dry weight over days via fermentation with mono culture cultivation	28
Figure 17	Biomass dry weight over days via fermentation with mixed culture cultivation	29
Figure 18	Biomass dry weight over days	29
Figure 19	Protein concentration over days via fermentation with mono culture cultivation	30
Figure 20	Protein concentration over days via fermentation with mixed culture cultivation	31
Figure 21	Protein concentration over days	31

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ABSTRACT

This project aims to formulate an effective microbial formulation for biodegradation of pineapple waste. A strain of fungus, *Trichoderma harzianum* UMAS SD17 and two strains of *Bacillus*sp., *Bacillus licheniformis* UMAS P7 and *Bacillus amyloliquefaciens* UMAS 1002 were used with varying co-cultivation in solid state fermentation where the pineapple waste are used as the substrate. Eight analytical analysis were done to determine the most efficient microbial formulation, which includes, protein content, carbon to nitrogen ratio, biomass dry weight, glucose and reducing sugar concentration and the level of cellulose, hemicellulose and lignin degradation. Based on the data obtained, the fermentation with co-cultivation of all three microbes shows the highest cellulose degradation from 37.65% to 12.93%, and the highest hemicelluloses degradation from 6.87% to 2.36%. It also shows the second highest rate of lignin degradation after formulation of *B.licheniformis* and *T.harzianum*, from 14.5% to 9.5%. After 24 days of fermentation, this formulation also shows the closest C:N ratio to the ideal compost ratio of 25:1 at 50.12:1, thus suggesting that this formulation of microbes consisting of *T.harzianum*, *B.licheniformis* and *B.amyloliquefaciens* is the most efficient formulation for biodegradation of pineapple waste and can be further used for a complete composting process.

Keywords : *Ananas comosus, Bacillus licheniformis* UMAS P7, *Bacillus amyloliquefaciens* UMAS 1002, *Trichoderma harzianum* UMAS SD12, Solid State Fermentation (SSF).

ABSTRAK

Projek ini bertujuan untuk membentuk formula mikrob paling cekap untuk proses biodegradasi sisa-sisa nanas. Satu jenis kulat, Trichoderma harzianum UMAS SD12 dan dua jenis bakteria Bacillus sp, Bacillus licheniformis UMAS P7 dan Bacillus amyloliquefaciens UMAS 1002 digunakan dengan pelbagai variasi ko-kultivasi dengan menggunakan kaedah fermentasi pepejal substrat di mana sisa-sisa nanas digunakan sebagai substrat. Lapan analisis analitikal dijalankan untuk menentukan formulasi mikrob paling berkesan, termasuklah, kandungan protein, ratio carbon kepada nitrogen, berat kering biojisim, peratusan glukosa dan gula, dan tahap degradasi selulosa, hemiselulosa, dan lignin. Berdasarkan data-data yang diperolehi, fermentasi dengan ko-kultivasi ketiga-tiga mikrob menunjukkan tahap degradasi sellulosa tertinggi dari 37.65% ke 12.93% dan degradasi hemisellulosa tertinggi dari 6.87% ke 2.36%. Formulasi ini juga menunjukkan tahap degradasi lignin kedua tertinggi selepas formulasi B.licheniformis dan T.harzianum, dari 14.5% ke 9.5%. Selepas 24 hari fermentasi, formulasi ini juga menunjukkan nisbah C ke N paling dekat dengan nisbah ideal baja iaitu 25:1 (50,12:1, dan dengan itu mencadangkan bahawa formulasi ini yang terdiri daripada B.licheniformis, B.amyloliquefaciens dan T.harzianum adalah formulasi paling cekap untuk biodegradasi sisa-sisa nanas.

Kata kunci : Ananas comosus, Bacillus licheniformis UMAS P7, *Bacillus amyloliquefaciens* UMAS 1002, *Trichoderma harzianum* UMAS SD12, Fermentasi pepejal substrat.

1.0 Introduction

According to a report by Performance Management and Delivery Unit (PEMANDU) under the Economic Transformation Programme (2012), Malaysia's pineapple industry are expected to have a potential Gross National Income (GNI) impact of 1.2 billion in 2020. Currently, pineapple industry generated an average of 56 million in export income annually, eventhough relatively, its industry is smaller compared to palm oil and rubber. Alongside the advancement of the industry, the by-product of the pineapple processing industry, as well as the by-product of domestic use of pineapple are increasing proportionally. These by-product are classified as pineapple waste. According to Buckle (1989), about 40%-50% of pineapple waste consists of fruit peels and its core. Due to the presence of high amount of cellulose in the pineapple waste composition, compared with other agricultural waste, its utilization are not given great attention to. These biodegradable elements and suspended solid caused a high biological oxygen demand and high pH in the soil (Kroyer,1991) and accumulation of these will adversely affects the environment. Apart from environmental effect, it will also be a wastage of all the organic material contained in the waste. Most of the organic material has the potential to be used to obtain a value added product or other beneficial substances.

Decomposition or bio-degradation is a natural biological process which include the physical breakdown of plant residues and the biochemical transformation of complex organic material of the dead material into simpler organic and inorganic material (Alexandra & Jose, 2005). By degrading the waste, its volume and quantity can be greatly reduced. This will helps in preventing environmental pollution and to obtain other potential useful products. In this project, the bio-degradation of the pineapple waste will be done via solid state fermentation (SSF) with co-cultivation of varying microbial formulations.

Two *Bacillus* sp., *B. licheniformis* UMAS P7 and *B.amyloliquefaciens* UMAS 1002, and *Trichoderma harzianum* UMAS SD12 are used in this project. These microorganisms are co-cultured with different formulation and are used in the bio-degradation of pineapple waste via solid state fermentation process and the efficiency of each formulation are compared. In order to measure the rate of the bio-degradation of the pineapple waste, 8 analytical measurement are used. The parameters are; lignin residual content, reducing sugar and glucose concentration, protein content, biomass dry weight, cellulose and hemicellulose content and carbon to nitrogen ratio. Each parameters are recorded periodically and are used as the basis for comparison to determine the most effective microbial formulation.

By inoculation and co-cultivation of different type of microorganisms in the bio-degradation of the waste, the rate of the degradation of the waste can be increased (hyphothesis). The main objective of this project is to evaluate various formulation of different co-cultivation between the microorganisms. Thus, by comparing the rate of the biodegradation, the most efficient microbial formulation can be formulated. To achive this aim, the following objectives are specified as follows :

Objectives :

i. To carry out solid state fermentation (SSF) in order to biodegrade the pineapple waste.
ii. To evaluate the effect of various formulation of co-culturing of different organisms to the rate of biodegradation of pineapple waste via Solid State Fermentation (SSF).
iii. To determine the most efficient microbial formulation in the biodegradation of pineapple waste via SSF.

2.0 Literature Review

2.1 Pineapple Industry and Its By-Product

In the 60s and the 70s, Malaysia are the top 3 pineapple producer with its main export market consisting of United States, Japan, Middle East and Singapore. In the late 90s, the export of canned pineapple decline while the export of fresh pineapple increases (Chan, 2000). By comparison of the market of canned and fresh pineapple, fresh pineapple market are relatively smaller and are targeted mainly to Singapore. However, the on-going research of plant biotechnology and the introduction of new hybrid are expected to continously boost the fresh pineapple market in the future (Chan, 2000).

In coherent with the developing pineapple industry, and also due to the 1997 haze problem in Southeast Asia that has caused a permanent banned on burning of agricultural waste, more emphasise are given to find alternative ways to manage the waste. Pineapple waste are the by-product of the pineapple industry processing which includes fruit peel and its residual pulp. Accumulation of these wastes that consists of high level of cellulose and hemicelluloses can pose danger to the environment.

Biodegradation of pineapple waste are one of the alternative to manage the by-product of the pineapple industry. Decomposition process are a natural biological process that occur naturally. It involve the physical breakdown of the waste and the bioconversion of complex organic matter into simpler organic and inorganic matter that have a beneficial properties towards the plant and the soil. It also produces a more stable and safe end product for disposal. Production of secondary metabolite such as citric acid or lactic acid can also be obtained from the biodegradation process via solid state fermentation technique.

2.2 *Bacillus licheniformis*

Bacillus licheniformis is found commonly in soil, manure and plant material . They are rod-shaped, gram-positive bacteria able to form spore under unfavourable condition. This bacterium are nutritionally very diverse and are able to produce a variety of extracellular enzyme. These two characteristics conbine with the spore-forming capability makes this bacterium suitable to be used in industrial scale of enzyme production. They are also important agents in nutrient cycling in soil. The optimum growth temperature of *Bacillus licheniformis* is 50^0C, while the optimum enzyme secretion temperature is 37^0C. Under harsh environment, it can survive by forming endospore and it can revert back to its vegetative state once the environment return to ideal state.

B.licheniformis is industrially used for production of alpha amylase (Bessler *et. al*., 2003). This bacterium is also used in the production of Bacitracin antibiotic that is used to inhibit its own growth. Bacitracin antibiotic will lyses the proplast of *B.licheniformis* with the presence of cadmium or zinc ion. In paper industry, this bacterium helps in the production of modified starch.

2.3 *Bacillus amyloliquefaciens*

Bacillus amyloliquefaciens are rod-shaped bacterium that are positive to catalase, gram-positive and are motile. They are known for their catabolic activities and their capability in breaking down complex macromolecules extracellularly. This characteristics makes them of medical significance and also suitable to be used in composting process.

As all bacteria species in *Bacillus* genus, they are commonly found in soil and are able to form endospore when the environment are unfavourable . This allows them to be

able to withstand harsh environment and subsequently making them suitable to be used in industrial scale. One of the extracellular enzyme produce by this bacterium are subtilisin, an enzyme capable of breaking down protein. Alpha amylase derived from this bacterium are also being used in starch hydrolysis. In textile industry, *B.amyloliquefaciens* are used to remove starch and also as additive for detergents.

2.4 *Trichoderma harzianum*

Trichoderma is a genus of common filamentous fungi that are unique due to its lifestyle and various interactions with other fungi, animal and plant . This genus are also able to antagonize plant-pathogenic fungi, stimulate growth of plant and defence responses. These characteristic makes them suitable to be used as biological control of plant diseases (Verena *et. al*., 2011). *Trichoderma* can be found mainly in soil and some may produce coconut-like odor. Its optimal temperature for growth is in the range of 25^0C-30^0C. At a temperature higher than this, the fungi will dies off. *Trichoderma* spp. are generally non-pathogenic to human, however, there are some infection cases reported in immunocompromise individual.

Trichoderma harzianum is a highly cellulolytic fungi that have a very rapid growth rate. Their cellulolytic characteristics makes it a highly efficient decomposition agent. In composting process, they acts as fungus activator. It hastens the decomposition of organic material especially the decomposition of lignocelluloses.

2.5 *Ananas comosus*

Ananas comosus are commonly known as pineapple due to its similar physical attributes to pine cone. Under the Bromoliaceae family, this species are the most economically significant. In Malaysia, apart from palm oil and rubber, pineapple are one of the agricultural-product major contributor to export earning with an approximately RM56 million profit annually. Ananas comosus has a cylindrical shape, with a crown-like rosette of leaves. Its skin's colour varies from yellow, green to brown. The yellow fibrous flesh near the base of the fruit has a more tender structure and more sugar content and thus sweeter.

2.6 Solid State Fermentation

Solid state fermentation is the process of cultivation of microorganisms on solid media with appropriate moisture level. The moist solid media can be either inert carriers or insoluble substance that in addition provide carbon as energy source for the microorganisms. Solid state fermentation can only be done in a small extend due to the engineering obstacle in building a large fermentor where all the parameter's including temperature, pH, oxygen level, and moisture gradient can be regulated with the present of limited water. However, the emergence of effective microbial strains developed via genetic engineering allows for the enzyme production via SSF to be done in industry scale. Even with the engineering problem, SSF is favoured over submerged fermentation (SmF) due to the simple technique involved, low level of catabolite repression and end product inhibition, less waste water output, better product recovery and high quality production (Ghildyal *et. al*., 1985).

Agricultural waste which consists of approximately 60-65% of starch capable to be hydrolysed to glucose are good resources for fermentation substrate. With the advancement of biotechnology field, utilization of agricultural waste to produce value added product has been continuously emerging. This will also directly helps in controlling pollution problem that their disposal might caused.

3.0 MATERIAL AND METHOD

3.1 Microorganism preparation

Trichoderma harzianum UMAS SD12, *Bacillus amyloliquefaciens* UMAS 1002 and *Bacillus licheniformis* UMAS P7 that are used in this project were obtained from Molecular Genetic Laboratory, UNIMAS. *B. licheniformis* P7 and *B. amyloliquefaciens* UMAS 1002 are maintained on nutrient agar at 37^0C (Sweetha, *et. al*., 2006) and *T. harzianum* SD12 are maintained on Potato Dextrose Agar (PDA) at room temperature until the entire agar is covered by the fungi. The working culture are stored at 4^0C.

3.2 Inoculum preparation

3.21 Bacterial inoculum preparation

For inoculum preparation, Luria broth were used. The broth are sterilized via autoclaving and allowed to cool down to room temperature. Two beakers of LB media are prepared for each *Bacillus*. Each nutrient media are inoculated with a full loop of both *Bacillus* respectively and both flask are incubated overnight on a shaking platform at 37^0C. After overnight incubation, the optical density of the bacterial suspension are checked via spectrophotometer at 600 nm (the desired OD is 1.0). The suspension are then centrifuged at 800rpm for 15 minutes. The supernatant are removed and the pellet was suspended in minimum salt media (MSM). The inoculum are stored at 4^0C for further used.

3.22 Fungus spore preparation

In order to harvest the spore from the Potato Dextrose Agar (PDA), 0.1% Tween 80 are used. 10 ml of the tween 80 are poured into the agar plate containing the fungi. The plate are then gently swirled. The resulting spore suspension are transferred into a sterile universal tube. The spores are counted under the microscope by using hemacytometer (the

desired spore suspension is 10^7 spores/ml). The inoculum were stored at 4^0C for further used.

3.3 Pineapple waste preparation

The pineapple waste were obtained from market place in Kuching. The waste are cut into small cube, boiled and dried in the oven at 60 ^{0}C overnight. The dried waste are grinded before being sieved to obtain the mesh size of 1mm.

3.4 Solid State Fermentation

This method was based on the method as described by Julio, Plinho, *et. al*. (2002), with slight modification. 5 g of the priorly prepared waste are placed in 250 ml Erlenmeyer flask. The waste are then sterilized via autoclaving. Mineral salt media are prepared and added to the flask. To determine the most efficient co-culture of microorganisms for biodegradation of the pineapple waste, 1 control set and 7 experimental set are set up consisting of different strain and combination of the *Bacillus* and *Trichoderma*. Each set are inoculated with different microorganisms as described in the Table 1. Each fermentation flask are then incubated at 37^0C for 24 days. Analysis are done every 6 days.

Table 1 : Experimental set with inoculation of different strain and combination of microorganisms

Experimental Set	Inoculation with *B.licheniformis* P7	Inoculation with *B.amyloliquefaciens* UMAS 1002	Inoculation with *T.harzianum* SD12
Control			
1	✓		
2		✓	
3			✓
4	✓	✓	
5	✓		✓
6		✓	✓
7	✓	✓	✓

3.5 Analytical Method

3.5.1 Biomass Dry Weight

All the flasks used for fermentation are weighed before and after the commence of the fermentation. To obtain the wet weight (ww), the difference between the weight of the flask with fermented material and the weight of the empty flask are calculated. 0.5 g of the fermented material was taken from each sample and dried until constant dry weight is achieved. The biomass dry weight are calculated as described in Table 2.

Table 2 : Biomass dry weight calculation

Wet weight (g)	Constant dry weight (g)	Amount of sample taken (g)	Biomass dry weight (g)
X	Y	0.4	$D_w = X \times \frac{Y}{0.4}$

3.5.2 Protein Concentration

The protein concentration were measured according to Bradford (1976) method. The protein are extracted using citrate buffer. To obtain the supernatant, the sample are centrifuged at 800 rpm for 20 minutes (Hafiza *et. al*., 2011). 10 ul of the priorly extracted protein are mixed with 1 ml of Bradford reagent (Amresco, USA) in a microcentrifuge tube. The mixture are then incubated at room tempererature for 3 minutes. The colour absorption are measured using UV spectrophotometer at 595 nm. A standard curve at 595 nm of absorbtion against Bovine Serum Albumin are prepared.

3.5.3 Carbon Content

Carbon content are measured using combustion method. 1 g of the fermented material are weighed and placed in a already weighed crucible. The fermented material are then dried

in the 80^0C oven until constant weight are achieved. The constant weigh are recorded. The fermented material are then combusted by using hot plate until it turns to ash. The weigh of the ash are taken and recorded. The carbon cntent are calculated by using the formula as described in Table 3.

Table 3 : Carbon percentage calculation

% organic matter	$\frac{\text{Oven dried weight} - \text{Combusted weight}}{\text{Oven dried weight}} \times 100$
Carbon content	% organic matter / 1.8

3.5.4 Nitrogen Content

Nitrogen content are measured using the standard Kjedahl method. Approximately 1 g of the fermented material are dried before beginning the process. The nitrogen content are calculated as follows;

% Nitrogen = (Titre volume x 0.1 x 1.4007) / sample weight (g)

3.5.5 Washing and Digestion Stage

The first stage in lignin residue measurement is the washing stage. The sample are mixed with double distilled water and centrifuged at 120 rpm for 1 hour. The water are then poured out and ethanol are added. The mixture are again centrifuge at 120 rpm for another hour. The ethanol are poured out and acetone are added. The mixture are then again centrifuge for 1 hour at 120 rpm. The excess acetone are poured out and the resulting washed material are left overnight in a fume chamber. On the next day, 0.4 g of the washed sample are weighed and digested with 70% sulphuric acid. The mixture are placed on 200 rpm shaker for 3 hours. After the digestion step, 250 ml of distilled water are added and the

mixture are autoclaved. This two stage are needed for the measurement of lignin, glucose and reducing sugar and calculation of cellulose and hemicellulose that will follows. The subsequent lignin, cellulose and hemicellulose measurement method are based on the method as described by Ververis *et. al.* (2007), with slight modification.

3.5.5.1 Lignin

After autoclave, the mixture are filtered suing a priorly weighed filter paper. The substrate that still remained on the filter paper is the insoluble lignin. The filter paper are dried in the oven overnight. On the next day, the filter paper with the filtered substrate are weight. The lignin residue are calculated as described in Table 4.

Table 4 : Lignin residue percentage calculation

Filter paper	Filter paper + Lignin	Sample weight	Lignin Weight	Lignin Percentage
X	Y	0.4 g	Y – X = Z	$\frac{Z}{0.4\ g} \times 100$

3.5.5.2 Reducing Sugar and Glucose

Reducing sugar and glucose are analysed using Di-nitrosalicyclic (DNS) method (Miller, 1959). The resulting liquid after lignin filtration was added with 50 ml of boiled distilled water. The pH are then adjusted to a pH in the range of pH 5 – pH 7. 2 ml of this test solution and 2 ml of the DNS reagent are mixed and heated at 100^0C for 15 minutes. The mixture are allowed to cool down to room temperature before the addition of 1 ml of 40% sodium tartrate (Rochelle) to stabilize the red-brown colour. The colour absorbtion are then measured using UV visible spectrophotometer at 575 nm. A standard curve at 595 nm of

absorbtion against glucose and xylose for glucose and reducing sugar standard respectively are prepared.

3.5.5.3 Cellulose and Hemicellulose Calculation

This calculation is based on method as described by Ververis *et. al.*, (2007).

% w/w cellulose content = (0.9 / 0/96) x C1 x (V / M) x 100 x d.f

% w/w hemicellulose content = (0.88 / 0.93) x (C2 – C1) x (V / M) x 100 x d.f

Where, C1 = Glucose concentration (g/L)

C2 = Reducing sugar concentration (g/L)

V = Total volume of sugar solution (L)

M = Dry weight of sample (g)

d.f = Dilution factor if any

4.0 Results and Discussion

4.1 Preparation of culture

4.1.1 *Trichoderma harzianum* culture

Trichoderma harzianum are cultured on Potato Dextrose Agar (PDA). Potato Dextrose agar are rich nutrient agar and are commonly used to culture fungi that feeds on decaying plant matter. PDA consists of potato infusion and dextrose. Throughout culturing process of the fungi, aseptic technique are applied to reduce the risk of contamination that could greatly affect the culture. The whole culturing process are done in laminar floor with sterilized blade. One important aspect to take note, in case of remelting the agar is needed, the potato dextrose agar should not be re-melted more than once as this can affect the agar quality. The *Trichoderma harzianum* are cultured for 6 days before sub-culturing are done. If the *T.harzianum* are left for a longer period of time, the fungi will turns green in colour. This will also occurs if the agar are exposed to sunlight.

The inoculum are prepared by using 0.1 % Tween 80. Tween 80 acts as surfactant to facilitate in the harvesting of spores. This process also needs strict aseptic condition to ensure no contamination occurs. The resulting spores suspension are placed in sterilized universal tube in 4 ^{0}C until further use. The spore are counted under microscope by using hemacytometer to obtain a final spore suspension of 10^{7} spores/ml. It is important to note that the spore suspension are only best used in the range of 1 to 2 weeks from the harvesting date to ensure the viability of the spores.

4.1.2 *Bacillus amyloliquefaciens* and *Bacillus lichniformis* culture

Both *Bacillus* sp. were maintained on nutrient agar (NA). Nutrient agar consists of beef broth and some yeast extracts. It is suitable to be used in student research as it does not